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Biochimica et Biophysica Acta 1737 (2005) 27 – 35
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Pulmonary surfactant function is abolished by an elevated proportion of cholesterol

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Received 26 April 2005; received in revised form 17 August 2005; accepted 6 September 2005
Available online 10 October 2005

Abstract

A molecular film of pulmonary surfactant strongly reduces the surface tension of the lung epithelium–air interface. Human pulmonary surfactant contains 5–10% cholesterol by mass, among other lipids and surfactant specific proteins. An elevated proportion of cholesterol is found in surfactant, recovered from acutely injured lungs (ALI). The functional role of cholesterol in pulmonary surfactant has remained controversial. Cholesterol is excluded from most pulmonary surfactant replacement formulations, used clinically to treat conditions of surfactant deficiency. This is because cholesterol has been shown in vitro to impair the surface activity of surfactant even at a physiological level. In the current study, the functional role of cholesterol has been re-evaluated using an improved method of evaluating surface activity in vitro, the captive bubble surfactometer (CBS). Cholesterol was added to one of the clinically used therapeutic surfactants, BLES, a bovine lipid extract surfactant, and the surface activity evaluated, including the adsorption rate of the substance to the air–water interface, its ability to produce a surface tension close to zero and the area compression needed to obtain that low surface tension. No differences in the surface activity were found for BLES samples containing either none, 5 or 10% cholesterol by mass with respect to the minimal surface tension. Our findings therefore suggest that the earlier-described deleterious effects of physiological amounts of cholesterol are related to the experimental methodology. However, at 20%, cholesterol effectively abolished surfactant function and a surface tension below 15 mN/m was not obtained. Inhibition of surface activity by cholesterol may therefore partially or fully explain the impaired lung function in the case of ALI. We discuss a molecular mechanism that could explain why cholesterol does not prevent low surface tension of surfactant films at physiological levels but abolishes surfactant function at higher levels.

Keywords: Captive bubble surfactometer; Atomic force microscopy; Pulmonary surfactant; Cholesterol; Dipalmitoylphosphatidylcholine (DPPC); Bovine Lipid Extract Surfactant (BLES)

1. Introduction

Pulmonary surfactant forms a molecular film at the air–water interface of the hydrated air–alveolar interface. Surfactant is secreted by the alveolar type II epithelial cells [1–3]. From its secretion form, lamellar bodies, surfactant evolves into tubular myelin and adsorbs to the air–liquid interface to form a tightly packed molecular film. This film reduces the surface tension to an equilibrium value of about 23 mN/m. As the area of the alveolar interface decreases upon expiration, the molecular film becomes compressed and the surface tension drops further. Direct measurements in the lung revealed a surface tension of almost zero at residual lung capacity [4]. This study also showed that upon inspiration, the surface tension might rise up to 30 mN/m at total lung capacity, and changes of surface tension of approximately 15 mN/m occur for deep breaths between 40 and 80% total lung capacity, but during tidal breathing, stays likely below 10 mN/m [5] (note that the surface tension of a free air–water interface is close to 70 mN/m at 37 °C). Both functional and dysfunctional surfactants can reduce the surface tension to about 23 mN/m, but only functional surfactants bring about the required further drop of surface tension close to zero upon film compression. Note that a surface tension of 23 mN/m throughout the lung would strongly impair lung function by reducing the gas-

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doi:10.1016/j.bbalip.2005.09.002

exchange area to about 1/3 of its normal size [5]. Pre-term infants do not have sufficient mature surfactant and as a result develop Respiratory Distress Syndrome (RDS) in a gestational age dependent manner. These neonates are treated by intra-tracheal administration of exogenous surfactant, spread from a bolus of a highly concentrated aqueous suspension. Notwithstanding the great success of this treatment, RDS remains one of the leading causes of the neonatal morbidity and mortality. This, and potential new applications in adults, such as treatment in the case of acute lung injury (ALI) make replacement surfactant formulations an active field of research.

Phospholipids are the primary surface tension lowering components in surfactant. Phosphatidylcholines (PC) represent 80% of its mass with up to half of the PC being the disaturated dipalmitoylphosphatidylcholine (DPPC); 5–10% by mass is the negatively charged phosphatidylglycerol (PG) [6]. DPPC is the most abundant molecule and is also the major component in any of the commercial surfactant replacement products [2,3]. In a pure film, DPPC permits surface tension reduction to near zero values upon compression. A significant amount of phosphatidylglycerols (PG) or, in some cases, phosphatidylinositols (PI) suggests an important role for either one or both of these negatively charged phospholipids as well [7,8]. PG and PI have been shown to improve the adsorption of surfactant to the interface.

In addition to its unique lipid composition, pulmonary surfactant contains two water-soluble (SP-A, SP-D) and two hydrophobic surfactant-associated proteins (SP-B, SP-C). The latter two are permanently associated with the lipids of surfactant because of their high amount of hydrophobic amino acid side chains. Mixed films that also contain SP-C and/or SP-B can sustain a very low surface tension as well as pure DPPC films. The proteins SP-C and -B efficiently recruit the lipids to the interface and promote more effective film organization. They are also responsible for the cohesiveness and mechanical strength of the film [8–17].

Cholesterol is present with 5–10% by mass (10–20 mol%) and constitutes the major fraction of the neutral lipids. The role of cholesterol has remained controversial. In a number of earlier studies, very low surface tension was not obtained in the presence even of physiological amounts of cholesterol [3,18–25]. These studies for the most part used incomplete surfactant mixtures or less than optimal test procedures. However, based on these findings, cholesterol is currently removed from most of the clinically used surfactant extracts and not added to artificial exogenous surfactants. But the stable near zero minimum surface tension has been found in the lung [26,27], making it evident that cholesterol at physiological proportions must not interfere with surfactant function in the healthy lung. In accordance with these findings, the work of Diemel et al. [28] showed no effect of cholesterol on the surface activity of an artificial surfactant of close-to-natural composition. More recently, others have observed similar results [29] and suggest that physiologically relevant amounts of cholesterol play an important role in surfactant function and should be investigated further.

To re-evaluate this disagreement on the functional role of cholesterol in physiological proportion, we studied the effect of cholesterol on the surface activity of pulmonary surfactant by adding cholesterol to a clinically used exogenous surfactant. BLES is a hydrophobic extract of bovine lung lavage, which differs from natural surfactant in the lack of SP-A and -D and cholesterol [20]. So as to better evaluate the role of cholesterol in vitro, an experimental protocol was followed that more closely resembles conditions in the lung. The captive bubble surfactometer (CBS) was used for measuring the surface activity, since in that device, in situ surface tension in the lung can be closely mimicked [26]. The CBS consists of a chamber, filled with buffered saline, and an air bubble floating against a convex agarose ceiling. Varying the volume in the chamber controls the volume of the bubble. As the volume is reduced, the surface area reduces and the surface tension of the bubble surface falls in the presence of a surfactant at the interface. Alterations in the surface tension at the air–liquid interface in the CBS are visualized as a change in the bubble shape from nearly spherical at high surface tension to more flat as the surface tension falls. Unlike in any other surface balance, the air–water interface in a CBS acts like a continuous surface. As a result, the interfacial film cannot escape the interface as long as the film does not collapse. This is important because surfactant films containing cholesterol have been shown to leak over barriers or spread along the restraining walls in other surface balances at high film pressure (i.e., low surface tension) [30,31]. The Pulsating Bubble surfactometer can suffer from film leakage as well though special measures can be employed to ameliorate this problem [32,33]. Thus, it is difficult to distinguish the effect of film leakage from that of dysfunctional surfactant when either technique is employed.

We also studied whether cholesterol interferes with surfactant function at higher-than-physiological proportion. Such interference has so far been investigated mainly with regard to serum proteins (for reviews, see [34,35]). But proteins alone have been found to have a very limited inhibitory effect on surfactant function [33,36]. Davidson et al. [37], looking at the very early stages of acute lung injury, reported a number of very rapid changes in the lung starting immediately after injury. These included increases in protein and large increases in cholesterol. Panda et al. [38] also reported an increased level of not just serum protein but cholesterol as well with respect to phospholipids in acutely injured lungs, as compared to normal lungs. The injured lungs were also distinct by a marked decrease in compliance indicating most likely a dysfunctional surfactant. Though the authors attribute this impairment primarily to blood serum proteins, they do note the possibility that the decrease in lung compliance could be due to excess cholesterol or to the action of proteins in combination with cholesterol. Lewis and Veldhuizen [35] also do not rule out a role for cholesterol in surfactant inhibition when they note that "Changes to neutral lipids have not been studied extensively to date".

In the current study, surfactants were administered to the air–water interface in a novel manner, developed in our lab and first employed in a study of bat surfactant [39]. The bubble's

air liquid interface was brought into contact with a small but highly concentrated aqueous bolus (27 mg/ml phospholipids with 0%, 5%, 10% or 20% by mass cholesterol added). The lower cholesterol amounts of cholesterol represent healthy lung levels and 20% cholesterol is the amount reported for injured lungs. We observed the change in the surface tension upon spreading and then upon a slow (quasi-static) change in area. Finally, the area was cycled as in normal breathing.

2. Materials and methods

2.1. Materials

BLES (BLES Biochemicals Inc, London Ontario see Yu et al. [20] for analysis of BELS composition) in non-buffered normal saline (pH 5–6) with a phospholipid concentration of 27 mg/ml was a kind gift by the manufacturer (BLES Biochemical Inc. of London, Ontario). Cholesterol was purchased from Sigma Chemicals, St. Louis, MO. A 1:1:1 ratio solution of methanol, chloroform, and BLES by volume was first vortexed and then spun at $100\times g$ for 5 min. The lower BLES/chloroform phase was removed and saved. Then second volume of chloroform was added to the methanol/water phase, which was then vortexed and spun like before. The methanol/water phase was discarded and the BLES in chloroform was removed and added to the saved BLES in chloroform, and then 0, 5, 10 or 20% of cholesterol (by mass) with respect to phospholipids in chloroform was added. Each solution was then dried under N2 and re-suspended in buffer (140 mM NaCl, 10 mM HEPES and 2.5 mM $CaCl_2$; pH 6.9) to obtain an aqueous suspension of BLES and cholesterol at a concentration of 27 mg/ml phospholipids. The samples with no extra cholesterol added were treated in the same way except that the final addition of chloroform before drying contained no cholesterol. Thus, the treatment required to add extra cholesterol would not likely be responsible for the differences found between samples with different amounts of added cholesterol. This same procedure was also used to add DPPC or DOPC to the BLES.

2.2. Surface activity assessment

The surface activity of the substance was determined using a laboratory-built, fully computer controlled CBS evolved from the apparatus described earlier [40]. The chamber of the CBS was filled with a buffer solution (140 mM NaCl, 10 mM HEPES, and 2.5 mM CaCl2, pH 6.9) containing 10% (by mass) sucrose, to increase the density of the buffer solution (1.04 g/ml) above that of the surfactant (1.01 g/ml). Approximately 0.05 μl of BLES–cholesterol mixture (27 mg/ml in buffer) was deposited at the concave agarose ceiling of the chamber by means of a transparent capillary. This allowed depositing a precisely defined volume of surfactant under visual control. The surfactant remained at the ceiling due to the density difference between it and the buffer solution in the chamber.

It has been shown earlier that sucrose has no effect on the surface activity of pulmonary surfactant [39]. To exclude a specific interaction of glucose with the surfactants used in the current study, the buoyancy of the subphase was changed by the addition of cesium chloride instead of sucrose as a control. The two ways of changing the density produced the same result (results not shown).

After inserting the surfactant into the chamber, a bubble, 2–3 mm in diameter, was allowed to float up to the ceiling. The shape of the bubble was imaged over time by a video camera (Pulnix TM 7 CN), the video frames were digitized by a frame grabber and saved to the computer's hard disk. During the experiment, the chamber was kept at 37 °C. Prior to evaluation of each sample, the chamber was cleaned by combination of water and methanol and tested to ensure that no surface-active contaminants were present.

A 5-min adsorption (film formation) period followed the introduction of the bubble in to the chamber during which the bubble was not manipulated and the change in surface tension over time was monitored. The chamber was then sealed and the bubble was expanded to a volume of 0.15 ml. Five minutes after the bubble was expanded, Quasi-static cycling commenced in the Quasi-Static portion of the experiment, the bubble size was first reduced and then enlarged in a stepwise fashion by altering the internal volume of the chamber. Each step had two components; a 3-s change in volume followed by a 4-s delay where the chamber volume remained unchanged and the film was allowed to "relax". There was a 1-min inter-cycle delay between each of four quasi-static cycles and a further 1-min delay between the Quasi-Static and Dynamic cycles. In the Dynamic Cycle portion of the experiment, the bubble size was smoothly varied over the same range as the last Quasi-Static Cycle for 20 cycles at a rate of 20 cycles/min. Bubble volume, interfacial area and surface tension were calculated using height and diameter of the bubble as described [41]. Tests were done with the Quasi-static cycles omitted and the Dynamic cycle results for samples with 0, 10 or 20% cholesterol were unaffected by this omission.

3. Results

3.1. Adsorption (film formation)

The fall in surface tension upon the adsorption of a surfactant is a measure of how fast this substance forms an effective film at the interface and so lowers its surface tension. With sufficient material, an equilibrium value will then be reached. Exogenous surfactant must be incorporated efficiently in the interface of a patient's lung, to ensure structural stability of the lung's alveolar system and prevent lung injury. All samples studied here spread efficiently in accordance with this requirement. The bubbles rapidly assumed a new shape after they rose to make contact with the surfactant at the agarose ceiling of the chamber. An equilibrium surface tension of approximately 23 mN/m was reached within a few seconds at most (Fig. 1). There was no difference in the behavior of surfactant samples with different amounts of cholesterol. In an attempt to avoid unphysiologically large excess of material at the interface, the volume of the bolus placed in the chamber was kept very small (~0.05 μl). Due to the small volumes involved, it was difficult to consistently place exactly the same amount of material at the agarose ceiling. Thus, the amount of matter deposited varied slightly from sample to sample. Furthermore, at the concentration chosen, the samples of surfactant formed granules, 20–150μm in diameter. A variety of phenomena suggest that these granules are particularly effective in film formation and that the surrounding matrix is minimally surface active (manuscript in preparation). The small volume deposited contained only a small and likely variable number of these surfactant granules. This variability in the deposited amounts of surfactant became apparent upon compression and expansion cycles and is described below.

3.2. Compression and expansion of the pulmonary surfactant films

In the lung, the molecular film of pulmonary surfactant must reduce the surface tension to almost zero upon the expiration. During tidal breathing, the change in relative interfacial area is small and the surface tension fluctuates between almost zero and certainly less than 20 mN/m. [4]. Hence, a functional surfactant must reach a surface tension close to zero upon compression in vitro and must not return to equilibrium when kept compressed. We found that surfactant films formed from BLES containing 0, 5 and 10% cholesterol by mass all fulfilled these requirements and did so both under quasi-static and dynamic compression and

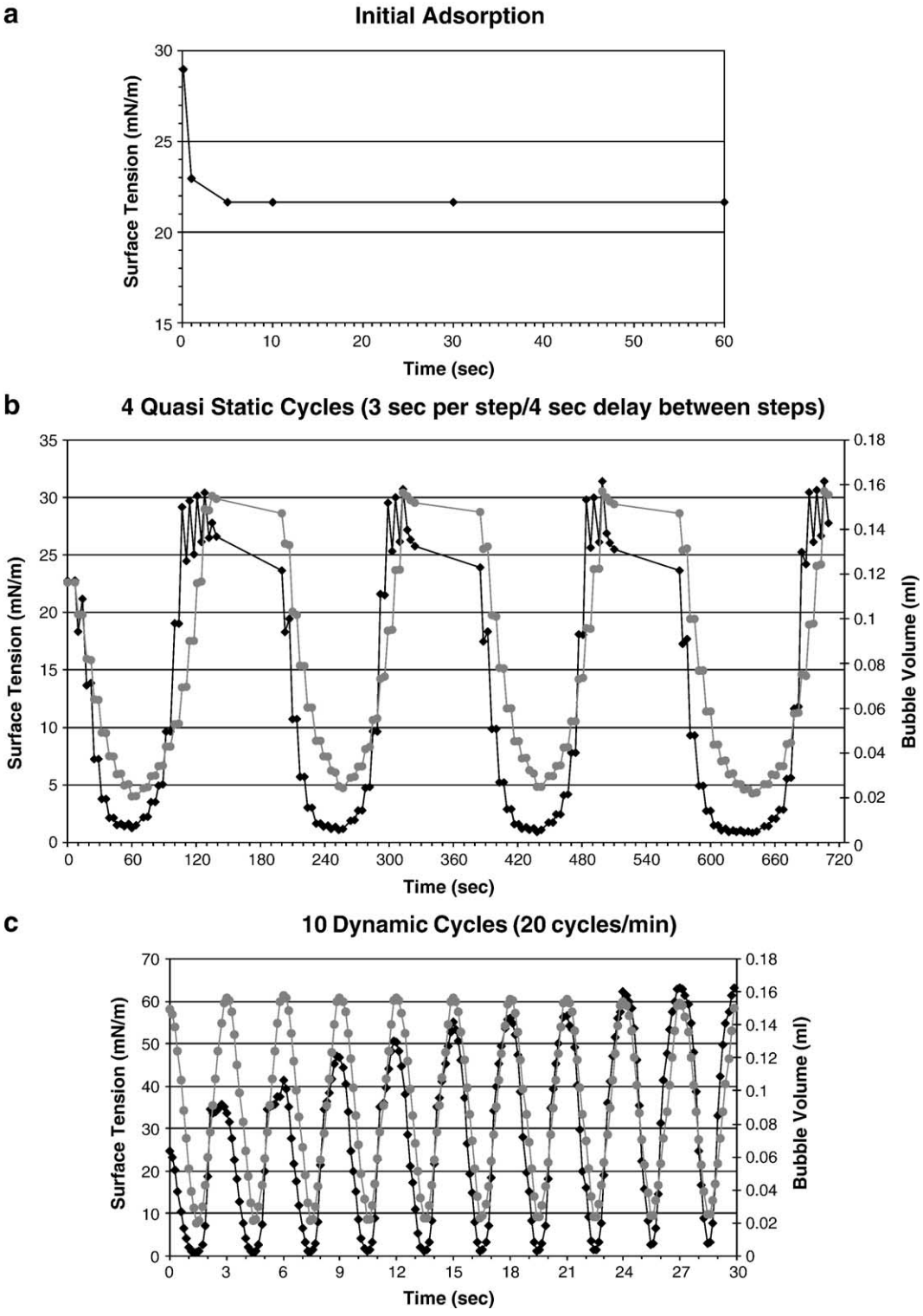

Fig. 1. Illustration of the experimental procedure. Plots of the typical changes in surface tension over time in the *adsorption* (a), *Quasi-Static Cycle* (b) and *Dynamic Cycle* (c) phases of a sample test. Measured bubble volume (grey lines) is plotted along with surface tension (black lines) in these last two graphs so that the changes in volume and the resulting changes in surface tension may be compared.

expansion. No significant difference in the behavior between these samples has been observed with respect to the minimal surface tension achieved (see Fig. 2. Note: for simplicity the 5% cholesterol curves are not shown, as they are indistinguishable from the 0% curves).

Under quasi-static conditions, the films were compressed stepwise and allowed to equilibrate between each step (Fig. 2 top). All samples containing less than 20% cholesterol reach a surface tension close to zero. A few samples show a highly compressible region of the area–surface tension isotherm at a

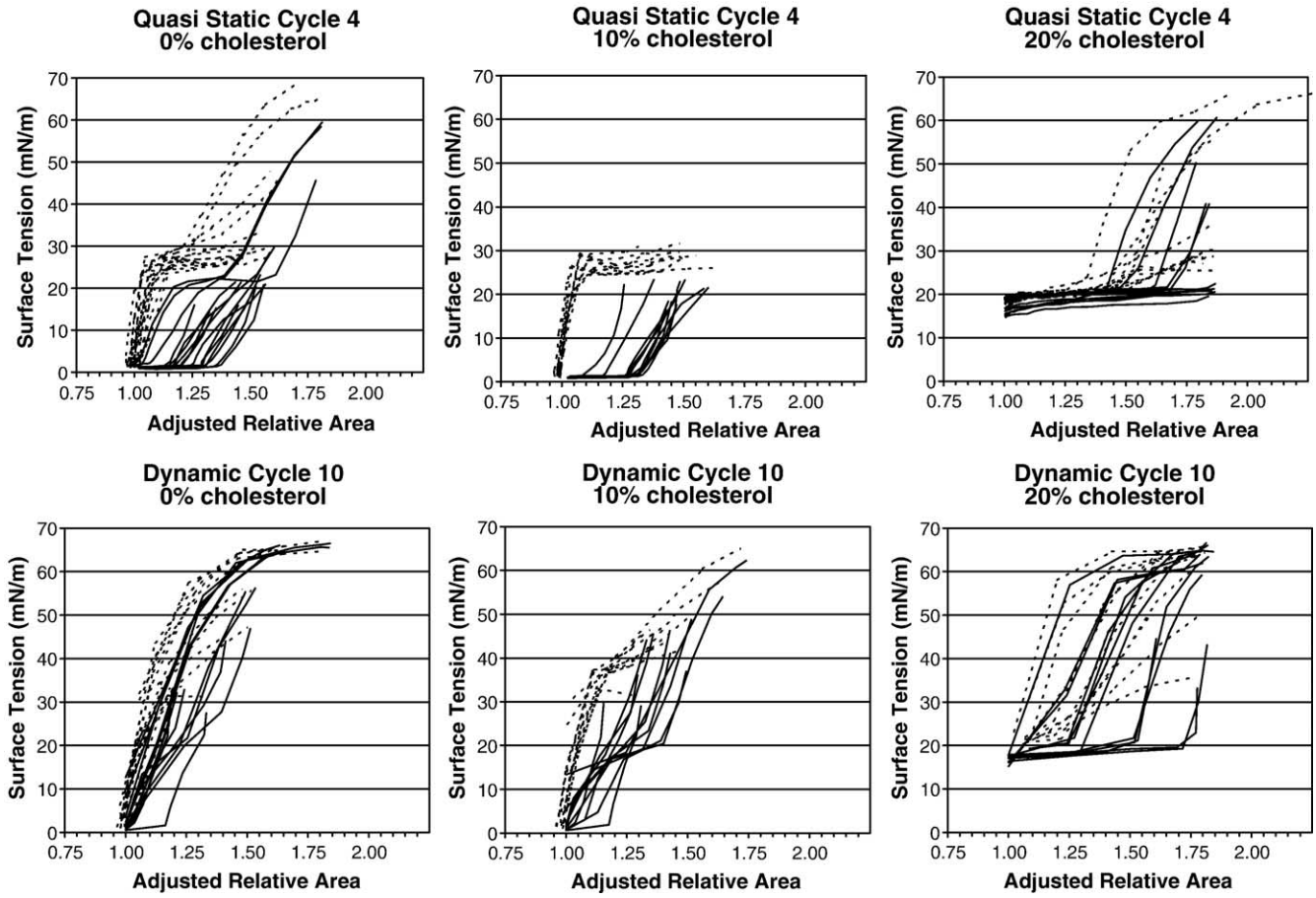

Fig. 2. The compression phase (solid line) and expansion phase (dashed line) of quasi-static and dynamic cycles are plotted for the samples having either 0, 10 or 20% cholesterol by mass. In the top row, the fourth of the repetitive quasi-static cycles (Q-stat) is shown (no significant difference between cycles occurred). The relative area has been normalized to be 1 at the end of the compression of the cycle. The bottom row shows the 10th of the series of repetitive dynamic cycles (no significant difference between cycles occurred). Each individual curve represents an individual sample.

surface tension of about 23 mN/m (plateau region of the area–surface tension isotherm) and then the isotherm dropped steeply to the near zero minimum values (<2 mN/m). For most samples the isotherm first fell rapidly to the near zero value and declined only slightly with further reduction in area. Upon expansion, the surface tension rises with little increase in area to just above the equilibrium value. A plateau where surface tension increases only slightly with increased area follows this rise. For some samples, this plateau is relatively short and is followed by a relatively steeply rising segment in the isotherm to well above equilibrium values.

During dynamic conditions, the films were compressed and expanded at a rate that reflected normal breathing (20 cycles per minute; Fig. 2 bottom). Under these conditions, the samples containing less than 20% cholesterol all attained the near zero minimum surface tensions achieved when compressed in a Quasi-static manner. Some samples' isotherms declined steeply from maximum to minimum surface tension while a number showed an inflection near equilibrium surface tension. In some cases, the inflection was followed by a less pronounced compression plateau while in the remainder the surface tension fell smoothly though less steeply to the near zero minimum. For a limited number of samples, the isotherm showed a small inflection near 23 mN/m and a truncated and slightly steeper plateau near minimum surface tension than was seen during Quasi-Static cycles. During Dynamic expansion the isotherms rose rapidly to above equilibrium surface tensions with very small increases in area.

The films containing less than 20% cholesterol could sustain a very low surface tension over an extended period of time. Following Quasi-static and Dynamic cycles, some of the samples were compressed to near zero surface tension and allowed to remain in that state for hours. During this time, the surface tension did not rise no matter whether the samples contained 0, 5 or 10% cholesterol.

Samples containing 20% cholesterol did not reach a low surface tension during compression. Those samples with above-equilibrium maximum surface tension behaved like the samples with lesser amounts of cholesterol when their surface tension was in this higher range. However, once the equilibrium surface tension of about 23 mN/m was reached, further film compression produced only a minimal reduction in surface tension below the equilibrium value for all samples. A minimum surface tension of about 16–20 mN/m was reached even after extensive compression. The minimum surface tension was not different for Quasi-Static and Dynamic cycles.

In summary, 5 (not shown) or 10% cholesterol by mass did not affect the most basic of pulmonary surfactant functions, that is, to reduce surface tension effectively and stably to a very low value. These samples behaved similar to samples containing no cholesterol. Samples containing 20% cholesterol did not reach low surface tension and so surfactant function was effectively abolished. In the following section, we discuss possible molecular mechanisms underlying the isotherms obtained with these samples and talk about the implications to human physiology and pathophysiology.

4. Discussion

4.1. Films, containing less than 20% cholesterol

Most samples reached equilibrium surface tension upon adsorption. A limited number of the samples showed maximum surface tensions well above equilibrium values. This indicated that the initial amount of surfactant was insufficient for a full coverage of the interface. Interestingly, most of these samples showed a plateau near equilibrium surface tension during Quasi-Static compression. We have shown earlier that a plateau in this region of the isotherm may be due to the formation of lipid double-layer structures adjacent to a molecular monolayer at the interface [42,43]. These structures have proven important to the stability of the interfacial film and are apparently a property of all functional pulmonary surfactant films [9,43]. That is, only after the interfacial film of pulmonary surfactant has developed the specific molecular architecture of planar regions, interrupted or surrounded by areas consisting of stacks of multi-layers, does it become incompressible down to a very low surface tension and adopt its unique mechanical stability. Since we adsorbed a minimal and variable amount of surfactant, in some cases, the surfactant film was initially in the low-compressibility region of the isotherm (a sufficient amount of surfactant was initially adsorbed to the interface), whereas in other cases, the distinct architecture of the film had first to be created by compression (in the case when an insufficient amount had been adsorbed). These findings are in line with our earlier findings that multi-layers may form either during the adsorption of the matter to the interface or later, upon compression of the film, if an insufficient amount has been adsorbed to the interface in the first place [42]. Whether the multilayer stacks have formed upon adsorption or upon the first compression had no influence on the function of the films and all films showed comparably low compressibility below equilibrium regardless of their behavior during the initial phase of the compression cycle. All but a few isotherms showed a plateau at low surface tensions and all showed a plateau in Quasi-static expansion after first showing a very steep rise at the start of expansion. This plateau again reflects the formation (compression) and unfolding (expansion) of multiple lipid stacks adjacent to a planar film and the width of the plateau regions of the isotherm was variable from sample to sample (see Fig. 2). The variability in the plateau width was not correlated to the amount of cholesterol present in the sample. Rather, it was most likely caused by the variability in the amount of matter, spread to the interface as described above. Despite there being some rather large area reductions after some of the samples reached near zero (<2 mN/m) surface tension values, this "overcompression" had no detrimental effect during the following cycles of expansion and compression. Rather, the slopes of the isotherms between the plateaus are quite similar both in the compression and expansion phase of the Quasi-static cycle and show no relation to the length of the plateau.

All samples containing less than 20% cholesterol also achieved the same near zero surface tensions during Dynamic cycles but generally reached much higher surface tension levels in expansion than found in Quasi-static expansion. The plateaus seen in Quasi-static cycles were either absent in Dynamic cycles or mostly replaced by more steeply sloped portions in the isotherm. Our explanation of the attenuated or missing plateaus is that during the more rapid Dynamic cycles, there is insufficient time for a complete unfolding of multilayers into a simple film at the air–liquid interface. As a consequence, a very high surface tension occurs at the end of the expansion cycle. It is notable that in the lung, such unphysiologically high surface tensions will not occur because the area change during normal breathing is much less than employed here.

4.2. Films containing 20% cholesterol

Samples with 20% cholesterol by mass behaved similarly to those with less or no cholesterol during the initial part of bubble compression when surface tension was above equilibrium. However, after achieving equilibrium, there was limited reduction in surface tension. During expansion, any increase in surface tension that did occur was observed only after notably greater increments in surface area. No restoration of the low-compressibility region of the isotherm was achieved with samples containing 20% cholesterol even after spreading a large excess of matter to the interface or compressing the film over a very large range (data not shown). In a physiological sense, this surfactant was dysfunctional. The isotherms suggest a continuous transition of a planar film into a multi-layered film upon compression and an unfolding upon expansion, without ever reaching a conformation, where the film becomes incompressible. Alternatively, multilayers may not have formed in the first place and matter was lost from the interface upon compression and then readsorbed upon expansion.

Films containing 20% cholesterol would not allow for structural stability of the lung because of the insufficient reduction of the surface tension. This breakdown of surfactant function at a higher proportion of cholesterol is very intriguing both for its implication for diseases and with respect to the molecular mechanism behind this phenomenon. Correlating our findings to the observations made by Panda et al. [38] strongly suggests that cholesterol is a contributing factor for surfactant inhibition in acutely injured lungs. Similar to the current study, approximately 10% cholesterol (mass) with respect to total surfactant lipids was associated with normal lung compliance and approximately 20% cholesterol was

associated with low lung compliance after acute lung injury. Panda et al. found protein level with respect to lipids was also increased in surfactant recovered from injured lungs. This leaves the possibility that proteins possibly in conjunction with cholesterol, originating most likely from blood plasma, were contributing to the inhibitory effect. There is reason to believe that plasma proteins by themselves may not have as marked an inhibitory effect on surfactant function as cholesterol alone (e.g.. [36] and as yet unpublished work by ourselves) though elevated amounts of proteins in combination with cholesterol may also be potent inhibitors of surfactant function.

4.3. How cholesterol interacts with the molecular components of pulmonary surfactant

Surfactant function in the current study deteriorated in an all or non-fashion with increased amount of cholesterol. Films containing 10% by mass of cholesterol or less all reached a very low surface tension (<2 mN/m), while all films containing 20% of cholesterol, in contrast, did not drop below 16 mN/m. To explain this behavior at the molecular level one needs to take into account the way cholesterol is incorporated within the molecular architecture of the surfactant film at a physiological level and at an elevated level. Cholesterol has a very distinct effect on the molecular arrangement of surfactant films even at low concentration as revealed by atomic force microscopy and fluorescence light microscopy [23,28,44]. For example, compression of films of purified calf surfactant phospholipids caused phase separation with large circular condensed domains of DPPC [45]. When cholesterol was added to these films, the size of the domains decreased and with further compression, apparent phase separation vanished and the films appeared homogeneous under a fluorescence light microscope. This major influence of the lateral distribution of surfactant components brought about by cholesterol was attributed to the reduction in line tension of the dividing line between the DPPC domains and the fluid matrix [45]. In their study, Discher et al. predicted from theoretical considerations that cholesterol should associate with DPPC rather than the more fluid matrix. This is highly plausible, as the planar cholesterol molecules of the plasma membrane are known to associate with the aliphatic groups of saturated lipids in sphingolipid cholesterol rafts. Tolerance of up to 10% cholesterol for functional surfactant and a breakdown of function above that proportion might be related to the saturation of the DPPC domains with cholesterol; i.e., cholesterol might not degrade the ability of the interfacial film to withstand a high film pressure as long as it is intercalated in-between the aligned, saturated aliphatic tails of DPPC. However, once exceeding the maximum possible stoichiometry of this complex and being forced to interact with unsaturated lipids or the surfactant protein C and/or surfactant protein B, cholesterol might abolish the ability of the film to withstand compression. To test the plausibility of this hypothesis, we increased the amount of DPPC in BLES containing 20% cholesterol such as to restore a physiological ratio of DPPC to cholesterol. As a result, the adverse effect of the high proportion of cholesterol was indeed reversed and eight out of ten of these samples reached a surface tension <2 mN/m upon compression (Fig. 3). To ensure that restoration of surfactant function was not because cholesterol was now merely diluted in a proportionally larger phospholipid fraction, we substituted the additional DPPC with DOPC as a control. Adding the same amount of DOPC to BLES containing 20% cholesterol did not restore surfactant function (Fig. 3) as only one sample reached near zero surface tension though three other samples did reach substantially lower levels than seen in samples with 20% cholesterol added. Note that DOPC, while otherwise identical to DPPC, has one unsaturated aliphatic tail and should therefore prevent efficient intercalation of cholesterol in the aliphatic tail region. Still, these materials did have some effect on surface activity of their own which affected a small proportion of the samples in each case. de la Serna et al. [29] provide suggestions as to possible mechanisms for these effects. As another control, increasing the propor-

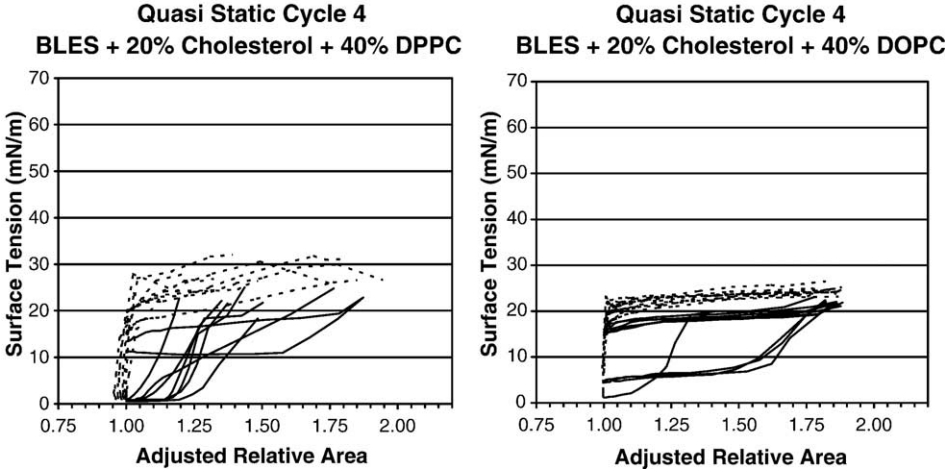

Fig. 3. Surface activity of BLES containing 20% cholesterol by mass after the addition of DPPC or DOPC. The amount of DPPC or DOPC added corresponded to 40% of the mass of the cholesterol-containing sample. The maximum natural molar ratio of approximately 1:2 (cholesterol/DPPC) has therefore been reestablished for these samples. The fourth of the repetitive quasi-static cycles (Q-stat) is shown. The relative area has been normalized to be 1 at the end of the compression of the cycle. Each individual curve represents an individual sample.

tional level of DPPC or DOPC in BLES without the addition of cholesterol did not affect surface activity of BLES in any way (results not shown). Together, these findings suggest that a physiological proportion of cholesterol is indeed accommodated in the surfactant film by forming a complex with DPPC. Assuming the correctness of this interpretation, it still remains an open question why cholesterol in a complex with DPPC should be benign with respect to surfactant function, whereas cholesterol not associated with DPPC abolishes surfactant function.

4.4. The role of physiological amounts of cholesterol

While no detrimental effect of physiological amounts of cholesterol has been found in this study, a beneficial function of physiological cholesterol levels within surfactant has not been identified, except for some special cases. Indeed, such function has been established for animals including bats and dunarts whose body temperature may change rapidly. For these animals, the level of cholesterol optimises surface activity of surfactant for the strongly changing body temperature to low values down to approximately 15 °C. [39,46,47]. Cholesterol also has a function in pinniped lungs' accommodate to the special requirements of deep diving [48]. It appears that the results, which showed a deleterious effect of cholesterol on the surfactant surface activity in that low minimum surface tensions were not achieved, were often obtained with simplified surfactant mixtures and almost always obtained in Langmuir troughs or in the pulsating bubble surfactometer without taking measures to minimize film leakage. It is conceivable that in these devices the cholesterol-containing films were able to spread along the restraining walls and barriers (film leakage) while similar films with no cholesterol did not. It should be noted that a difference in film leakage would not be due to a mere difference in surface viscosity. Films of BLES with physiological amounts of cholesterol added to them are not different with respect to surface viscosity from films containing no cholesterol [38,49]. Nevertheless, an increased ability of cholesterol-containing surfactant films to spread over restraining walls in the Langmuir trough might reflect a property related to the spreading of surfactant films evenly across the highly structured lung with its tiny cavities and narrow airways.

5. Conclusion

The current study establishes that cholesterol at physiological proportions has no detrimental effect on the surface activity of pulmonary surfactant, notwithstanding its marked influence on the molecular architecture of the interfacial films formed. A beneficial function of physiological amounts of cholesterol in the human lung, however, has yet to be discovered. However, recent studies have indeed established a role for cholesterol in other mammalian species such as bats [47] pinnipeds [48] and other species [50].

At an elevated level, cholesterol abolishes one of the lung surfactant's most important functions, that is, the ability to reach near zero surface tension. This finding may bring about a change in paradigm how surfactant inhibition is explained and, eventually, treated in the case of acutely injured lungs and possibly in other conditions with impaired surfactant function as well. Until now, surfactant inhibition has largely been attributed to factors other than cholesterol. Clearly, the role of cholesterol in normal lung function and in conditions where surfactant function has been compromised requires careful re-evaluation with the best methodologies currently available.

Acknowledgements

This research has been supported by CIHR (Canadian Institute of Health Research) and ACHF (Alberta Children's Hospital Foundation). We are grateful to Dr. S.U. Hasan for valuable discussions and also to BLES Biochemicals Inc, London Ontario for generously providing BLES.

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